REPORT DOCUMENTATION PAGE

Form Approved

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time f and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarc information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for informa 1204, Arlington, VA 22202-4302, and to the Office of management and Budget, Paperwork Reduction Project (0704-0188)

AFRL-SR-BL-TR-98-
0445

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYF
	June 1995	Final

4. TITLE AND SUBTITLE
Binding of Helocobacter pylori to Human Gastric Mucosa: Identification and Characterization of a Lewis b Bingind Protein

5. FUNDING NUMBERS

6. AUTHORS
Susan D. Biegel

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR/NI
110 Duncan Avenue, Room B-115
Bolling Air Force Base, DC 20332-8080

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for Public Release

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*
See attached.

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

19980519 093

DTIC QUALITY INSPECTED 3

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

ABSTRACT

For nearly fifty years, it has been well known by epidemiologists that individuals expressing the O blood group phenotype have a higher risk of developing gastric and duodenal ulcers than individuals with A or B blood group phenotypes. Because the etiology of the disease was not understood, this phenomenon remained a mystery for decades. With the isolation of a Gram-negative, spiral shaped bacterium from human gastric mucosa, *Helicobacter pylori*, came the answers to many questions concerning peptic ulcer disease, including this one. Recently, it has been shown that the Lewis^b (Le^b) blood group antigen, expressed both on blood cells and gastric epithelium, acts as a specific receptor for *H. pylori*. Le^b is the major blood group antigen in the Lewis system expressed in individuals with the O phenotype. In individuals of the A or B blood group phenotype, this epitope is usually modified with either a terminal GalNAcα1.3 or Galα1.3, respectively. The lectin-like specificity of the host cell:bacterial cell interaction strongly implies that a Le^b-specific adhesin is present on the surface of the microorganism.

We aim to identify and characterize this adhesin not only to gain valuable knowledge of the pathogenesis of *H. pylori*, but because of its role as a potential vaccine candidate. In the course of this three year study, we plan to purify the Le^b-specific adhesin, clone and mutagenize the gene encoding it, and assess its priority as a possible vaccine candidate. We also plan to evaluate transgenic mice for their use as an animal model for Le^b-dependent colonization of gastric epithelia.

Binding of *Helicobacter pylori* to Human Gastric Mucosa: Identification and Characterization of a Lewis^b Binding Protein

Susan D. Biegel

Preliminary Examination
Department of Microbiology and Immunology
University of North Carolina
Chapel Hill, NC
June 26, 1995

ABSTRACT

For nearly fifty years, it has been well known by epidemiologists that individuals expressing the O blood group phenotype have a higher risk of developing gastric and duodenal ulcers than individuals with A or B blood group phenotypes. Because the etiology of the disease was not understood, this phenomenon remained a mystery for decades. With the isolation of a Gram-negative, spiral shaped bacterium from human gastric mucosa, *Helicobacter pylori*, came the answers to many questions concerning peptic ulcer disease, including this one. Recently, it has been shown that the Lewis^b (Le^b) blood group antigen, expressed both on blood cells and gastric epithelium, acts as a specific receptor for *H. pylori*. Le^b is the major blood group antigen in the Lewis system expressed in individuals with the O phenotype. In individuals of the A or B blood group phenotype, this epitope is usually modified with either a terminal GalNAcα1.3 or Galα1.3, respectively. The lectin-like specificity of the host cell:bacterial cell interaction strongly implies that a Le^b-specific adhesin is present on the surface of the microorganism.

We aim to identify and characterize this adhesin not only to gain valuable knowledge of the pathogenesis of *H. pylori*, but because of its role as a potential vaccine candidate. In the course of this three year study, we plan to purify the Le^b-specific adhesin, clone and mutagenize the gene encoding it, and assess its priority as a possible vaccine candidate. We also plan to evaluate transgenic mice for their use as an animal model for Le^b-dependent colonization of gastric epithelia.

SPECIFIC AIMS

Helicobacter pylori is now thought to be the major cause of peptic ulceration and acute gastritis, as well as a risk factor in the development of gastric adenocarcinoma. While treatment of *H. pylori* infection has come a long way in the past several years, many treated individuals revert back to an infected state. There is also the fear that antibiotic resistant strains will arise due to selective pressure. Additionally, asymptomatic carriers of *H. pylori*, who would not receive treatment against the organism, do have an increased risk of gastric adenocarcinoma. For these reasons, it is very important to study the organism so that preventative measures against infection can be developed.

One important virulence factor that many pathogenic organisms possess is the ability to bind the host. It has been elegantly shown that *H. pylori* specifically binds the Lewis^b epitope of cells of the human gastric mucosa. The bacterial component responsible for this interaction has not yet been identified. This component may be an important virulence factor for *H. pylori*, and so it is important to consider its role in pathogenesis. In order to begin this process, we plan to accomplish the following aims.

I. Purification of a Lewis^b binding protein from *H. pylori*

II. Antibody analysis

a. Does antisera from infected individuals recognize the adhesin?

b. Does rabbit antisera made specifically against the purified adhesin possess bactericidal activity or block bacterial binding?

III. Cloning and mutagenesis of the Lewis^b binding protein gene from *Helicobacter pylori*

IV. Evaluation of the colonization of *H. pylori* in the transgenic mouse model and effects of the Lewis^b-specific adhesin mutant in this system

BACKGROUND AND SIGNIFICANCE

In 1984, a species of microaerophilic, Gram-negative, spiral shaped bacteria was isolated from human gastric mucosa (Marshall and Warren, 1984). This organism was classified as *Campylobacter pyloridis* and the name was later changed to *Helicobacter pylori*. Since its isolation, *H. pylori* has been implicated as the etiological agent of peptic ulcer disease. The organism is found in greater than 90% of peptic ulcer (gastric and duodenal) patients (Cerda and Fowler, 1994), while its prevalence in the general population is much lower in developed countries (Talley, 1993). Preliminary evidence also exists linking *H. pylori* to gastric adenocarcinoma (Parsonnet, 1993).

Several putative virulence factors of *H. pylori* have been identified to date. These factors include a urease, flagella, a mucinase, a cytotoxin, and two adhesins. The urease is believed to aid in bacterial survival of the harsh gastric environment by generating ammonia from urea to neutralize the low pH (Segal *et. al.*, 1992). The ammonia production may also be partially responsible for host-tissue damage (Hazell and Lee, 1986). The sheathed flagella and the mucinase are thought to be important in the colonization of gastric epithelia. The flagella confer a high degree of motility to the organism, which is necessary to penetrate the viscous mucous lining to reach a host cell (O'Toole et. al., 1994). The mucinase possesses an activity which breaks down the mucous layer to allow rapid penetration. This breakdown of the mucous layer may also contribute to ulcer formation by exposing the epithelium to gastric acid (Smith *et. al.*, 1994). The vacuolating cytotoxin causes the formation of acidic intracellular vacuoles in gastric epithelial cells. This vacuolization inevitably leads to host-cell death (Cover and Blaser, 1992). The proton pump inhibitor, Bafilomycin A1, blocks the cytotoxin mediated vacuolization in HeLa cells, suggesting that the cytotoxin either acts as a proton pump, or acts to regulate a eukaryotic vacuolar proton pump (Papini *et. al.*, 1993). Lastly, two putative adhesins have been identified in *H. pylori*: a lipid-binding adhesin and a sialic acid-binding adhesin. The lipid-specific adhesin most likely recognizes phosphatidylethanolamine, gangliotriaosylceramide, and gangliotetraosylceramide. Phosphatidylethanolamine (PE) is detectable in the human stomach antrum, so it is possible for this to be a receptor for *H. pylori*. Furthermore, *H. pylori* does not bind well to tissue culture cells lacking PE (HEL cells) (Lingwood *et. al.*, 1993). Evans *et. al.* (1993) reported the cloning of a sialic acid-specific adhesin (*hpa*A). It is unlikely, however, that this "adhesin"plays a role in the colonization of the gastric epithelium for several reasons: (1) there were no detectable sialylated glycoconjugates on tested gastric mucous cells; (2) *H. pylori* binding was not inhibited by incubation with sialylated glycoconjugates (Falk *et. al.*, 1993); and (3) An *hpa*A mutant is still able to bind gastric epithelia (P. Falk, personal communication).

It has been known for nearly half a century that individuals expressing the O blood-group phenotype have an increased risk of developing duodenal ulcers (Clarke *et. al.*, 1955). It has also been shown that these individuals are at higher risk for gastric ulcers (Mentis *et. al.*, 1991). Until very recently, nobody has been able to develop a model to explain this phenomenon.

The Lewis^b (Le^b) blood group antigen is expressed on gastric-surface mucous epithelial cells as well as blood cells, mainly in individuals of the O blood-group

phenotype. Individuals with A or B blood-group phenotypes modify most copies of this antigen with either a terminal GalNAcα1.3 or Galα1.3, respectively (Boren *et. al.*, 1994) (Figure 1). Boren *et. al.* (1993) have elegantly shown that the Lewis^b blood group antigen acts as a specific receptor for *H. pylori.* This binding is optimal when bacteria are in the stationary phase of growth.

The evidence supporting a Lewis^b receptor for *Helicobacter pylori* is overwhelming. Boren *et. al.* (1993) reported that the Le^b antigen chemically linked to albumin inhibits the binding of *H. pylori* p466 to human gastric mucous cells expressing Le^b up to 93% after incubation with the bacteria. Additionally, when human gastric mucosa sections were pre-incubated with monoclonal antibodies against Le^b, bacterial binding was inhibited 79%. Not surprisingly, they also reported that *H. pylori* did not bind human gastric mucosa not expressing Le^b.

Furthermore, they show that the binding is highly specific for Le^b, and not other related antigens. To show that this is a very specific binding, they created a panel of albumin-linked fucosylated carbohydrate chains, including Le^b, H-1, H-2, Le^a, Le^x, and Le^y. As previously mentioned, the Le^b-neoglycoprotein was able to inhibit bacterial binding to gastric epithelial cells almost completely. Only one other glycoprotein was able to even partially mimic this; the H-1 glycoconjugate was capable of reducing *H. pylori* binding 48%. The other glycoconjugates had no effect on bacterial binding. This indicates that the Galβ1.3Glc linkage of the type 1 chain is important and that a terminal fucosyl residue is essential for the adhesin:receptor interaction to occur, with the branched fucosyl residue aiding the interaction (Figure 1). These results strongly suggest that a highly specific interaction between the Le^b antigen and the bacterium occurs and that the interaction is not due to a non-specific interaction such as charge. Because of the specificity of this interaction, we are convinced that *Helicobacter pylori* expresses a Lewis^b adhesin on its surface.

Falk *et. al.* (1995) have recently developed a transgenic mouse that further supports the Le^b receptor model and is a potential animal model for studying *H. pylori* adherence and infection *in vivo*. They engineered a mouse line with an α-1,3/4-fucosyltransferase fused to a pit cell lineage specific promoter. This allows for the expression of the Le^b epitope in the gastric pit/surface mucous cells in the transgenic mice. Clinical isolates of *H. pylori* bound to the gastric epithelia of these mice *in vitro*, but not to that of their non-transgenic littermates. As expected, pre-incubation of the bacteria with soluble Le^b blocked binding. Showing *in vivo* colonization of *H. pylori* in these mice will be critical in assessing its use as a tool for evaluating therapeutic and vaccine strategies.

This document will describe the manner in which we propose to identify the Le^b-specific adhesin and define its role in the pathogenesis of *H. pylori.* The NIH is actively seeking to fund grant proposals investigating various aspects of *H. pylori* pathogenesis including:

a) identification of virulence factors of the organism and the effect of a deletion of the virulence factor gene on pathogenesis

b) use of animals that can be infected with human isolates of *H. pylori* and are amenable to evaluation of prophylactic and therapeutic strategies

c) vaccine development

We feel that the work proposed here will either achieve, or bring us closer to achieving these very important goals.

RESEARCH DESIGN AND METHODS

Specific Aim I. Purification of a Lewis^b binding protein from *H. pylori*

It is clear from the evidence presented that *Helicobacter pylori* almost certainly expresses an Le^b-specific adhesin. It is not clear, however, whether this adhesin is a single monomeric protein or is composed of multiple subunits. Many examples exist of adhesins that are thought to be monomeric proteins, including the glycolipid-binding adhesin from *Neisseria gonorrhoeae* (Paruchuri *et. al.*, 1990) and the lipid-binding adhesin from *H. pylori* (Lingwood *et. al.*, 1993). The classic example of an adhesin which is made up of multiple subunits is the pyelonephritis associated pilus of uropathogenic *E. coli*. The pilus is made up of several subunits including the structural subunit, PapA, and the subunit responsible for mediating the specific interaction with the eukaryotic receptor, PapG. Each individual pilus is composed of approximately 10^3 helically arranged PapA subunits. The actual "tip adhesin", PapG, is located at the distal end of the pilus as a single or low copy subunit (Lindberg *et. al.*, 1987; Lund *et. al.*, 1987). It is generally agreed that pili are not produced by *H. pylori* and that such structures are not responsible for attachment to host cells (M. Huesca, L. Bry, T. Trust, personal communications). This does not, however, rule out the possibility that the adhesin may be composed of multiple subunits.

In order to isolate the *H. pylori* Le^b-specific adhesin, an affinity purification scheme will be used. Presumably, the adhesin is associated with the bacterial outer membrane so the first step will be to isolate membrane proteins. Since the adhesin will later be purified on an affinity column, it is absolutely critical that at least a partially native protein structure is maintained at this step.

Membrane fractionation and solubilization of proteins. *H. pylori* will be grown under microaerophilic conditions (5%O_2, 10%CO_2, 85%N_2) at 37° C on *Brucella* agar containing 10% bovine blood and 1% IsoVitalex for five days (Falk *et. al.*, 1993). This will ensure that the bacteria have reached stationary phase and optimal Le^b binding activity. Isolation of total cellular membrane proteins will then be achieved as follows. Bacteria will be suspended in Tris-EDTA buffer and washed twice. They will then be lysed by passage through a French Pressure chamber three times at 15,000 lb/in^2. Unbroken cells will be removed by a brief centrifugation, and the supernatant will be subjected to centrifugation at 100,000 x g for one hour. The supernatant will be removed, and the pellet should contain the total cellular membrane fraction. The membranes will not be further fractionated into inner and outer membranes, as this step is unnecessary for our purposes and could result in the loss of the adhesin into the inner membrane fraction, especially if it is a lipoprotein (C. Cornelissen, personal

communication).

Disassociation of the adhesin from the membrane fraction while retaining a semi-native conformation may prove difficult. In order to determine the optimal conditions for adhesin solubilization, several different detergents at different concentrations will be employed. The specific detergents and the detergent concentrations used must be strong enough to allow the disassociation of the adhesin from the membrane, but mild enough to allow it to keep a native configuration. We will initially select the mild detergents recommended by Hjelmeland and Chrambach (1984) (CHAPS, octyl glucoside, and Triton X-100) at concentrations of 0.1%, 1%, and 5% in phosphate buffered saline (PBS). Pelleted membrane preparations will be incubated with continuous rotation with each detergent at the different concentrations for one hour at room temperature. Following incubation, the samples will be centrifuged at 100,000 x g for one hour and the supernatant containing solubilized membrane proteins (S100) will be collected. This will be repeated and the solubilized protein from both incubations will be pooled.

Binding inhibition assay. In order to determine the optimal detergent at the optimal concentration for solubilization, an assay must be designed to determine whether the adhesin had retained enough of its native configuration to allow for affinity purification. To do so, we will adapt the approach that Boren *et. al.* (1993) initially used to show bacterial binding to Le^b. Either 1μg of Le^b conjugated to human serum albumin (HSA) or 1 μg of HSA-Le^a (negative control) will be spotted in duplicate onto nitrocellulose filters using a dot blot apparatus. The filters will be blocked with BSA for one hour. One of the duplicate membranes will be further "blocked" with the solubilized membrane solution for four hours. *H. pylori* strain p466 will be labeled with digoxigenin-3-*O*-succinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (DIG-NHS; Boehringer Mannheim), as described by manufacturer's instructions. This labeling has previously been shown not to interfere with the specific interaction of *H. pylori* with the Le^b antigen (Boren *et. al.*, 1993). The labeled bacteria will be added to the membranes at an OD_{600} of 0.1 and will be incubated two hours at room temperature. The membranes will then be washed six times in PBS to remove unbound bacteria. Filters will then be incubated with anti-DIG antibody conjugated to alkaline phosphatase for one hour followed by washes in PBS to remove unbound antibody. Lumi-Phos (Boehringer Mannheim, IN) will be added, filters will be exposed to X-ray film, and bacterial binding will be measured by chemiluminescence. Binding of bacteria to the Le^b antigen should be blocked by the solubilized membrane fractions if the adhesin is in a soluble, native form. If bacterial binding to Le^b is not blocked, then the adhesin was either not solubilized sufficiently, or the detergent was too harsh and the adhesin was denatured.

Affinity purification of the Le^b adhesin. Affinity purification of the *H. pylori* Le^b binding protein will be achieved by the method described by Saadi *et. al.* (1994) for the isolation of the $Lewis^a$-specific adhesin from *Staphylococcus aureus*. Purified $Lewis^b$ antigen conjugated to human serum albumin (HSA) will be purchased from Accurate Scientific, Westbury, NY and will be covalently bound to Synsorb affinity absorbent (ChemBiomed Ltd., Edmonton, Alberta, Canada) as described by the

manufacturer. Since the binding site of Le^b consists of carbohydrate and not protein, one need not be concerned about denaturation during coupling to the Synsorb beads. To ensure that the Le^b antigen was indeed coupled to the beads, a small amount of the Synsorb-Le^b slurry will be incubated with Le^b-specific monoclonal antibody (obtained from Accurate Scientific, NY). If the antibody is not eluted by washing with PBS, then it can be assumed that the coupling was successful.

The *H. pylori* solubilized membrane preparation will be added to the HSA-Le^b-linked beads and rotated on a Nutator at four degrees overnight. The slurry will be centrifuged at 50 x g for five minutes and will be washed twice with PBS containing the detergent concentration previously determined, to remove unbound protein. Bound material will be eluted in PBS plus detergent containing 2% ammonia and will be collected in the supernatant following brief centrifugation. The eluate will be immediately neutralized and dialyzed against PBS plus detergent. Alternatively, the bound material could be eluted by dropping the pH, followed by immediate neutralization and dialysis. The beads will be washed once with PBS plus detergent and the eluate from that wash will be pooled with the previous. Following elution, the protein concentration will be determined using the BCA assay (BioRad). A small amount will be analyzed by SDS polyacrylamide gel electrophoresis (PAGE) to ensure that the purification was successful. This eluate will be run side by side with pre-affinity membrane material and also the unbound material collected after incubation with the HSA-Le^b-linked beads. The expected result is that the unbound material will have a very similar protein profile to the pre-affinity membrane material, but with a decrease in the relative amount of the putative adhesin (or adhesin subunits).

As a negative control to ensure that the putative adhesin is not in fact displaying affinity for the Synsorb bead, but for the $Lewis^b$ antigen, a mock affinity purification will be carried out using Synsorb beads that have not been linked to HSA-Le^b. The expected result for this control will be that all of the protein will be washed off in the initial PBS washes, and nothing will be eluted in the ammonia wash. Additionally, affinity chromatography occasionally leads to false positive results where a specific interaction does not take place, but actually an interaction due to ionic charge (Phizicky and Fields, 1995). To control against this occurrence, an additional control will be performed. Le^a shares a similar ionic charge with Le^b but has been shown not to bind *Helicobacter pylori* strain p466. We will therefore perform a mock affinity purification using HSA-Le^a-linked Synsorb beads to ensure that the binding of the putative adhesin is not due to charge alone. This will also control against purification of proteins with affinity to HSA.

Because affinity purification often carries along low levels of contaminating protein, the eluate containing the putative adhesin will be run over a gel filtration column for size fractionation. All protein-containing size fractions will be tested for *H. pylori* binding inhibition activity, as measured by the previously described binding inhibition assay. Alternatively, fractions could be spotted onto nitrocellulose and probed using iodinated HSA-Le^b. Fractions containing either binding inhibition activity or that bind iodinated HSA-Le^b, should contain highly purified *H. pylori* Le^b-specific adhesin. A positive result in either of these assays will also suggest that a partially native protein structure is maintained. Gel filtration size standards will be

run so that we will be able to estimate the sizes of fractions containing Le^b-binding activity.

If multiple size fractions display Le^b-binding activity, it could mean that the adhesin is composed of multiple subunits, with the size variations being due to breakdown or variations in the number of structural subunits added to the adhesin during formation. To investigate the possibility of an adhesin complex further, the size(s) of purified native adhesin, as determined by gel filtration, will be compared to the size(s) of band(s) visible by SDS PAGE. If the denatured adhesin appears to be the same size as the native adhesin, it is likely that the "holo-adhesin" is a monomeric, single subunit protein. If one or more proteins are observable that are all considerably smaller than the native adhesin, then it is likely that the holo-adhesin is a homo- or hetero-multimeric structure, respectively.

For cloning purposes, the N-terminal amino acid sequence of the adhesin monomer or adhesin subunit will be determined. Protein to be sequenced will be prepared as described by Yuen *et. al.*, 1989. The samples will then be sent to ImClone Systems, NY, for N-terminal sequence analysis by Edman degradation. If proteins are N-terminally blocked, they will be trypsin digested, size fractionated by HPLC, and internal sequence will be determined by Edman degradation. Knowledge of the N-terminal or internal amino acid sequence will allow for the design of degenerate oligonucleotides for use in cloning, and may also shed some light on the nature of the adhesin by examining it for homology to other known adhesins. This kind of knowledge could also effect the ways in which we go about cloning the adhesin.

Feasibility and Alternatives: As previously stated, solubilization of the adhesin from the membrane fraction in a native concentration is not trivial, but certainly not impossible. The detergents that we have selected have been described as mild detergents that are useful for our specific purpose. The reason for using various concentrations for each given detergent is that concentrations that are too high lead to loss of function, while concentrations too low are not sufficient for solubilization (Hjelmeland and Chrambach, 1984). If none of these predicted detergents are successful, others may be employed.

I am confident that the affinity purification of the $Lewis^b$-specific adhesin will be straightforward, as we will be utilizing the same technique employed for the purification of the *Staphylococcus aureus* Le^a-specific adhesin (Saadi *et. al.*, 1994). If the adhesin is a multisubunit structure, however, there is the possibility that the holo-adhesin would not be retained throughout the purification procedure. Even if this were to be the case, we are quite confident that will will be able to purify the subunit(s) responsible for the Le^b-specific binding due to the nature of affinity chromatography.

It is possible that we may not be able to obtain extremely high yields of the affinity purified protein by the scheme described. If this were to be the case, we would utilize the small amount of purified protein obtained to determine the N-terminal amino acid sequence. With this information, we will be able to clone the gene as described in Specific Aim III. Once the gene is cloned, it can be fused to a histidine tag, and large amounts the recombinant 6xHis:Le^b-specific adhesin could be purified

by histidine-tagged affinity chromatography (Hoffmann and Roeder, 1991). This purified protein could then be used for antibody preparation.

Specific Aim II. Antibody analysis

It is clear that an immune response is mounted against *H. pylori* in infected individuals as shown by the presence of specific antibodies against the vacuolating cytotoxin and the *cagA* gene product (Cover *et. al.*, 1993; Blaser *et. al.*, 1995). Whether antibodies against a particular antigen are produced in infected individuals and whether antibodies produced against the purified antigen are detrimental to the colonization or survival of the pathogen are critical questions to ask in considering the antigen as a vaccine candidate.

Subaim II a. Does antisera from infected individuals recognize the adhesin?

Helicobacter pylori is an interesting pathogen in that although a host immune response is indeed mounted against it during colonization and infection, the organism can still persist in that host for decades. Adhesins are often dominant surface antigens on pathogens. For vaccine related purposes, it would be useful to see if antibodies recognizing the Le^b-specific adhesin are produced in infected individuals. This may shed some light on whether the adhesin is expressed *in vivo*. It will also be interesting to see if differences in expression of anti-adhesin antibodies in infected individuals correlates with clinical symptoms.

Serum from infected individuals displaying either no symptoms, chronic active gastritis, gastric ulceration, duodenal ulceration, or gastric adenocarcinoma and from control, non-infected individuals will be obtained from the North Carolina Memorial Hospital, Chapel Hill, NC. These antisera will be tested for reactivity against purified Le^b-specific adhesin by the enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene 96-well microtiter plates will be coated with a mixture of native and denatured purified *H. pylori* Le^b-specific adhesin by the method described by Harlow and Lane (1988). Purified adhesin will be bonded to the microtiter wells by merely incubating it at room temperature in a humid atmosphere for two hours without the use of any harsh chemicals or detergents. By this procedure, approximately 100 ng of protein should adhere per well. After the adhesin is bound and the wells are washed, remaining sites for protein binding on the polystyrene will be blocked with BSA for two hours. Serum from each individual will be added to an adhesin-coated microtiter well. Adhesin-specific serum antibodies will be allowed to adhere for 2 hours at room temperature in a humid atmosphere, and unbound antibodies will be washed off. To detect the presence of adhesin-specific antibodies bound to the antigen, commercially available rabbit-antihuman IgG antibody conjugated to alkaline phosphatase will be added to the microtiter plates and allowed to bind for two hours. Unbound antibody will be washed away and the phosphatase substrate, p-nitrophenyl phosphate, will be added. If phosphatase is present, then the substrate will be hydrolyzed to produce the colored product, p-nitrophenol. The formation of this product is therefore an indirect measure of the amount of immunoreactive antibody in the serum, and can be measured spectrophotometrically at 405 nm. A positive ELISA reading would indicate that the adhesin is expressed *in vivo* in the particular patient. A negative result would not necessarily mean that the adhesin is not

expressed *in vivo*. It is possible that the adhesin is just not expressed at a time when the bacteria are available for phagocytosis by antigen presenting cells. Perhaps the adhesin is only expressed once in a protected environment such as the thick mucous layer of the gastric pit. Alternatively, a negative result could mean that adhesin peptides are non-immunogenic. A positive control for the ELISA will be performed using the affinity purified adhesin-specific antiserum described below.

By testing serum from infected patients displaying diverse manifestations of *H. pylori* infection, we may be able to gain some knowledge of the importance of the adhesin for different symptoms. While it is likely that the adhesin is expressed in bacteria isolated from individuals displaying all clinical manifestations of *H. pylori* infection, it would be interesting to know if its expression could be correlated with certain disease manifestations.

Subaim IIb. Does antisera made specifically against the purified adhesin possess bactericidal activity or block bacterial binding?

For a vaccine candidate to be plausible, antibodies against it must be detrimental in some way to the pathogen. Blocking bacterial colonization and bactericidal activity are two ways in which this can be achieved. Specific antisera against the Le^b-specific adhesin will be necessary to examine these possibilities. It may also prove useful for cloning purposes.

Preparation of affinity purified antisera. Pre-immune serum will be taken from four New Zealand white rabbits and will be stored for use as controls in various experiments. Rabbits will be subcutaneously injected in four spots with 20 μg of purified Le^b-specific adhesin emulsified in TiterMax (Vaxcel) adjuvant (according to manufacturer's instructions), followed in two weeks by re-injection of antigen emulsified in half as much TiterMax adjuvant. After two more weeks they will be further re-injected with antigen in PBS alone. Two weeks following, the rabbits will be bled and the serum separated by centrifugation. Post immune sera will be affinity purified against a mixture of native and denatured Le^b-specific adhesin to eliminate serum antibodies not specific for the adhesin that may cross-react against *H. pylori*. This should also rid the serum of complement. A mock purification of the pre-immune sera will be performed in exactly the same manner. The presence or absence of adhesin-specific antibodies will be confirmed by ELISA against a mixture of native and denatured adhesin as previously described. The specificity of the antisera will be evaluated by Western blot using *H. pylori* whole cell lysate as antigen. The Western blot analysis will not show if antibodies to the native adhesin were made, but it will confirm that antibodies are specific and that the affinity purification was successful. For cloning purposes (Aim III), Western blot analysis will be performed using *E. coli* DH5αMCR whole cell lysate as antigen to ensure that the antisera do not cross-react with proteins of the cloning strain.

Since specific antibodies produced against the adhesin could differ from rabbit to rabbit, both the bactericidal studies and binding inhibition studies will employ antisera produced from several rabbits.

Bactericidal Activity. Antibody-dependent complement-mediated

bactericidal killing is an effective mechanism by which a host can be protected against potential pathogens. This occurs by the classical complement pathway as shown in Figure 2. If an antigen has the ability to induce bactericidal antibodies in an animal model, its value as a potential vaccine candidate is strongly increased. For this reason, we will evaluate the ability of the *H. pylori* Le^b-specific adhesin to induce the production of bactericidal antibodies against *H. pylori* in a rabbit model.

Preparation of complement. Complement will be prepared from pooled normal human serum (PNHS) from individuals with no history of *H. pylori* infection. The PNHS will be preabsorbed with glutaraldehyde-fixed *H. pylori* strain p466 in a manner analogous to that described by Joiner *et. al.* (1983). The purpose of this is to deplete the serum of any cross-reactive antibodies against *H. pylori.* This absorbed PNHS will be evaluated at the North Carolina Memorial Hospital to ensure that it contains normal complement levels.

Serum bactericidal assay. The serum bactericidal assays will be performed basically as described by Joiner *et. al.* (1985). Bacteria will be suspended to 1500 cfu/ml in Hanks' buffered salt solution (HBSS). They will then be pre-sensitized with purified post-immune sera or pre-immune sera (negative control) by incubation for 20 minutes at 37° C. The complement source will then be added followed by continued incubation at 37° C for one hour. Bacteria will then be plated and incubated as previously described. Colonies will be counted and colony forming units of bacteria incubated with post-immune and pre-immune sera will be compared. Significant killing will be defined as less than 50% viability of bacteria exposed to post-immune serum compared to bacteria exposed to pre-immune serum. An additional control where bacteria will be incubated only with either active complement or with complement that has been heat inactivated for 30 minutes at 56° C, will be performed and bacteria will be plated. Viable colony forming units following these treatments will be compared. If viability is lower for the bacteria treated with active complement, it could be due to bacterial killing by the alternate (antibody-independent) complement pathway (Figure 2). This would indicate that a portion of the bacterial killing in our experimental assays is due to this alternative pathway as well. If so, it should not alter our analysis of the antibody-mediated killing by complement, as presumably the antibody-independent killing would be equal in experiments using post- or pre-immune sera and thus would subtract out. If killing by the alternative pathway is extremely high, however, it could mask killing by the classical, antibody-dependent pathway. While it is unlikely, if this does pose a problem, the complement source could be absorbed with antibody against an alternative pathway-specific complement component such as Factor B (obtained from Quidel), thus blocking the alternative, antibody-independent pathway.

Blocking of bacterial binding. Inhibition of bacterial binding is another way in which antibodies can be protective against disease. We plan to determine whether affinity purified rabbit antisera against the Le^b-specific adhesin is capable of blocking binding of *H. pylori* p466 to purified $Lewis^b$ and to human gastric epithelial cells expressing the Le^b epitope.

To determine whether antibody directed against purified adhesin is capable of blocking bacterial binding to purified Le^b, we will use an assay based on similar

principles as the binding inhibition assay described in Specific Aim I. As described previously, 1μg of HSA-Le^b will be spotted onto nitrocellulose using a dot blot apparatus and filters will be blocked with BSA. In this assay, rather than determining whether solubilized protein blocks the binding site of immobilized Le^b (the eukaryotic receptor), we will look at whether antibodies raised against the bacterial adhesin block binding. DIG-labeled bacteria will be incubated with the affinity purified antisera for one hour at room temperature and will be washed once to remove unbound antibody. The bacteria will then be added to the filter spotted with immobilized Le^b antigen and will be rocked for two hours at room temperature. Detection will proceed using alkaline phosphatase-conjugated anti-DIG antibody as previously described. Controls will include using no antibody source as a positive control for binding, and using "affinity purified" pre-immune sera instead of post-immune sera to control for effects of contaminants in purified immune serum. Additionally, all experiments will be performed in duplicate, using Le^a rather than Le^b to control for non-specific binding due to charge.

If antisera directed against the adhesin is capable of blocking binding to purified, immobilized Le^b, we will investigate whether the antisera can block binding to human gastric epithelial cells expressing the $Lewis^b$ epitope. We will do this by using the general *in situ* adherence assay described by Falk *et. al.* (1993). Uninfected human stomach samples expressing Le^b will be obtained from the Department of Pathology at Washington University. Tissues will be fixed in 10% formalin and embedded in paraffin. Stationary phase *H. pylori* will be suspended in a sodium chloride/ sodium carbonate solution and will be incubated with 0.1 mg/ml fluorescein isothiocyanate (FITC) for one hour at room temperature in the dark. Following this incubation, the bacteria will be washed three times in PBS/ 0.05% Tween 20 to remove unbound FITC. Labeled bacteria will then be incubated with the affinity purified antisera for one hour at room temperature and washed to remove unbound antibody. Tissue sections will be deparafinized and will be rinsed in PBS and then blocked with BSA for 30 minutes. The labeled bacterial suspension will be diluted and incubated on the tissue section for one hour. The sample will be washed six times to remove unbound bacteria and will be viewed by fluorescent microscopy. Positive controls for bacterial binding will be bacteria incubated with "affinity purified" pre-immune sera, or incubated with no serum at all. The negative control for binding will be to use gastric epithelial cells that do not express Le^b.

There is always the concern in binding assays whether pre-labeling the bacteria will interfere with its binding capability. Labeling bacteria with either DIG or FITC should not interfere with the binding of *H. pylori* to either immobilized Le^b or to human gastric mucosa, as this has previously been shown not to be a problem (Boren *et. al.*, 1993; Falk *et. al.*, 1993). Furthermore, it is unlikely that this labeling will interfere with the interaction of adhesin-specific antibodies with the adhesin, as this interaction likely takes place at a site in close proximity to the site responsible for binding to the eukaryotic receptor (which we know to be unaffected).

Analysis. Showing that a particular antigen is capable of inducing antibodies in an animal model that are bactericidal or block the binding of a pathogen is a positive indication that the antigen may be a successful vaccine candidate. If the

antigen does not induce production of such antibodies, however, it does not necessarily mean that the antigen would not be a successful vaccine, as the antibodies produced in the animal model may not be identical to those produced in humans. On the other hand, the converse is true. Just because antibodies made in rabbits have these activities does not mean that human antibodies will share the activities. Additionally, for a vaccine to be successful, the antigenic species must be exposed and available on the surface of the pathogen. Also, the organism itself must come into contact with the antibodies in vivo. This may be an important issue with *Helicobacter* since it is fairly well protected from antibodies in the gastric mucous layer. It may be important for antibody to find it before it reaches the gastric mucosa. This may pose a problem if the adhesin was only expressed under conditions encountered in the gastric mucosa. In this case, we would be dependent on the production of acid resistant secretory antibodies.

Specific Aim III. Cloning and mutagenesis of the Lewis^b binding protein gene from *Helicobacter pylori*

The most conclusive way to prove that the putative Lewis^b-specific adhesin is indeed responsible for the binding of *H. pylori* to human gastric epithelial cells would be to make a mutant incapable of expressing the adhesin and showing that the mutant is rendered unable to bind the eukaryotic cells. We will describe two approaches that could be taken in order to obtain such a mutant. Both involve cloning the adhesin gene (either the whole gene or part of it), inserting an antibiotic resistance marker into the gene, and then crossing it back into the *H. pylori* chromosome by allelic replacement. The difference between the two approaches lies in the screening for the positive clone. The first approach will rely on the expression, membrane localization, and native conformation of the cloned adhesin. The second approach, while less rapid, will not be dependent on any of these factors.

Cloning Approach #1. Because of the rapid nature of the first approach, it is the approach of choice. Since the success of this approach will be completely dependent on the adhesin maintaining a native configuration in the membrane of the *E. coli* cloning strain, it will only be attempted if we have reason to believe that the adhesin is composed of a single gene product in a monomeric form.

Cloning. *H. pylori* chromosomal DNA will be isolated as described by Leying *et. al.* (1992) and will be subjected to incomplete digestion using the restriction enzyme *Sau*3A I. *Sau*3A I cuts most DNA frequently because it recognizes a palindromic sequence of only four base pairs. Since the digestion will be incomplete, it is highly likely that at least one fragment will contain the whole Lewis^b-binding gene (*lbb*A). The digested DNA will then be ligated into the compatible *Bam*H I site behind the T7 promoter of the inducible expression vector pET3 (New England BioLabs, Inc.). By using this expression system, we will not have to rely on expression from the native *H. pylori* promoter. The ligation mixture will be transformed into *E. coli* strain BL21, which harbors a plasmid that encodes the T7 RNA polymerase behind the *lac* promoter. Positive transformants will be selected in Luria broth containing 30 μg/ml chloramphenicol and 100μg/ml ampicillin.

Enrichment. Because we are attempting to isolate a clone from an entire

genomic library, it would be very helpful to enrich for the positive clones in some way to reduce the number of colonies that will need to be screened. We will do so by a modified version of the "panning" method described by Paruchuri *et. al.* (1990) for the isolation of the *Neisseria gonorrhoeae* glycolipid-binding adhesin gene. Pure HSA-Le^b will be immobilized onto a nitrocellulose filter. The filter will be blocked with 3% bovine serum albumin in PBS for one hour. The BSA solution will be poured off and 10 ml of a 5 x 10^8 cfu/ml suspension of the recombinant *E. coli* will be incubated with the membrane for four hours with continuous rotation at room temperature. Unbound *E. coli* will be removed by washing four times with PBS. Bound recombinants will be scraped off the filter and suspended in Luria broth containing ampicillin and chloramphenicol. These recombinants will be grown to 5 x 10^8 cfu/ml and the enrichment process will be repeated two more times. The recombinants from the final enrichment will be resuspended and plated onto Luria-ampicillin/chloramphenicol plates. As a negative control, this enrichment will be performed in duplicate with non-recombinant *E. coli* to ensure that it does not bind to Le^b.

Isolation of positive clone. If the enrichment was successful, then almost all recombinants should contain the full length adhesin clone. Twenty colonies will be picked and whole cell lysates will be prepared by boiling for five minutes in SDS. The lysates will be separated by SDS PAGE and transferred to nitrocellulose. Western blot analysis will then be performed using antiserum derived against the purified Le^b-specific adhesin described in Aim II. Clones of various insert size expressing the adhesin will be stored in freezer medium at -70° C and the nucleic acid sequence of the insert of the smallest size, as measured by gel electrophoresis, will be determined by dideoxy nucleic acid sequencing as described by Sanger *et. al.* (1977). The open reading frame (ORF) encoding the adhesin will be determined by matching the deduced amino acid sequence to the actual N-terminal amino acid sequence of the purified adhesin (or adhesin subunit). The gene encoding the $Lewis^b$-binding protein will be termed *lbbA*. The plasmid containing the smallest fragment encoding the adhesin will be termed pSB1.

The enrichment process proposed is an extremely powerful tool that, if successful, will greatly reduce the number of transformants to be screened. Notably, it was tremendously successful for the cloning of the *Neisseria gonorrhoeae* glycolipid-binding adhesin gene. After only three rounds of enrichment, all of the recombinants tested were found to contain the open reading frame ultimately determined to encode the adhesin (Paruchuri *et. al.*, 1990). Because of the ease of this enrichment protocol and past success with it for cloning a bacterial adhesin, we put great faith in it. The caveat of this enrichment scheme, however, is that the adhesin must be expressed on the outer membrane of the cloning strain in an active conformation. Because no in depth studies concerning protein localization and export in *H. pylori* have yet been reported, there is no precedence to indicate whether or not the cloned Lbb1 will be localized to the outer membrane of *E. coli* or maintain a functional configuration. If the enrichment scheme does not yield clones that express Lbb1, we will assume that either it was not expressed, or it was not localized or assembled correctly in the outer membrane of the cloning strain of *E. coli*. If this is the case, then the enrichment scheme will have to be abandoned.

Cloning Approach #2. This second approach will be taken if we have reason to believe that the adhesin is a multimeric structure, or if the enrichment process is unsuccessful due to any of the reasons mentioned above. Abandoning the enrichment scheme would mean that many more recombinants would need to be screened. While this would be disappointing, it would certainly be doable. The screening process could be done relatively rapidly by utilizing the standard hybridization colony lift format described by Sambrook *et. al.* (1989). Thousands of colonies per 138 mm filter can be screened by this method, which is critical since several thousand colonies may need to be screened.

The first step of this cloning strategy will be to generate a size selected genomic library of *H. pylori*. This will aid greatly in reducing the number of clones to be screened. Construction of the size selected library will be accomplished by digesting *H. pylori* chromosomal DNA with various restriction enzymes. Southern blot analysis will be performed on the digested DNA, probing with ^{32}P- labeled degenerate oligonucleotides that will be designed based on the N-terminal (or internal) amino acid sequence of the purified adhesin or adhesin subunit. The stringency of the hybridization will be such that only a single band will be visible when exposed to X-ray film. The DNA fragment that hybridizes to the probe should contain at least the 5' end of our gene of interest (if the oligonucleotide was based on the N-terminal amino acid sequence). This analysis should also ensure that there is only one copy of the gene in question. The enzyme that is selected for subsequent cloning will be one that cuts the DNA such that the 5' end of the putative adhesin gene is on a reasonably sized fragment for cloning (approximately 0.5-7.0 kb) and that cuts the cloning vector, pACYC184, in a unique site. Microgram quantities of *H. pylori* DNA will be digested and electrophoresed on a preparative gel. A section of the gel corresponding to the size fragment containing the 5' end of the adhesin gene will be cut out of the gel, and the DNA will be extracted. A small amount of this DNA will be examined by Southern analysis to ensure that the DNA fraction actually contains the correct fragment. This size selected DNA will be ligated into a compatible site in pACYC184 and transformed into *E. coli* DH5αMCR. Transformants will be plated onto Luria agar containing the appropriate antibiotic for selection.

After transformation into *E. coli* DH5αMCR and selection on Luria-antibiotic plates, colonies will be lifted onto nylon filters and probed with the degenerate oligonucleotides previously described. The stringency of the hybridization reaction will be such that hybridization to wild type *H. pylori* but not *E. coli* occurs. The approximate number of colonies to be screened will be as follows:

$$\textbf{\# colonies to be screened} = \frac{\textbf{size of genome}}{\textbf{size of fragment}} \times \textbf{\% of DNA in fraction} \times 3$$

Colonies that hybridized to the probe will be picked and their plasmid DNA will be sequenced to confirm the clones. This approach will not ensure us a full-length clone, and so we will determine by Western analysis whether the adhesin is expressed. One distinct advantage of this approach is that a gene product would not necessarily need to be made for it to be successful. This would be critical if the adhesin is lethal to *E. coli* in multiple copy. Whether or not a gene product is made, a null mutation could be obtained in the manner described below.

Mutagenesis. In order to convincingly show the role of Lbb1 (Lewis^b-binding protein) in colonization, it will be necessary to obtain an Lbb1 null mutant of *H. pylori*. Regardless of the cloning strategy used, the mutagenesis strategy will be the same. An Lbb1 mutant will be constructed by insertion of a kanamycin-resistance cassette (*aphA*) into the cloned putative *lbbA* open reading frame followed by allelic replacement of the mutated gene into the chromosome of *H. pylori*. The location of the open reading frame will be deduced by dideoxy nucleic acid sequence analysis (Sanger, *et. al.*, 1977), as will a suitable restriction site for the insertion.

The plasmid containing the partial or complete adhesin gene will be linearized with a restriction enzyme that recognizes a unique site in the putative coding region of *lbbA*. If no compatible ends flank the kanamycin-resistance cassette, both the linearized plasmid and the kanamycin-resistance cassette will be treated with Klenow to generate blunt ends. The resistance marker will be ligated to the plasmid, and will be transformed by the standard calcium chloride method into *E. coli* strain BL21 or DH5αMCR and selected on Luria agar containing 30 μg/ml kanamycin and the antibiotic(s) necessary for selection of the vector (and plasmid containing the T7 polymerase gene if BL21 is used). A null mutation will be confirmed by the absence of the adhesin in whole cell lysate as analyzed by Western blot if the adhesin was expressed in the original clone, otherwise the mutation will need to be confirmed by nucleic acid sequence. The plasmid with the insertional mutation in *lbbA* will be termed pSB2.

The parent plasmid of pSB2, either pET3 or pACYC184, is incapable of replication in *Helicobacter pylori* (H. Mobley, personal communication). Therefore, if pSB2 is transformed into *H. pylori*, and the transformants are selected on media containing kanamycin, the colonies that grow should have arisen by homologous recombination of the insertional copy of *lbbA* into the wild type site on the chromosome. Therefore, pSB2 will be isolated and desalted and will be electroporated into *H. pylori* strain p466. Although *H. pylori* is known to be naturally transformation competent, transformation by electroporation is the method of choice because of its higher efficiency (H. Mobley, personal communication). Following revival on Brucella agar supplemented with bovine blood and IsoVitalex under microaerophilic conditions for 24 hours, the putative transformants will be scraped off, resuspended, and plated onto Brucella agar containing bovine blood, IsoVitalex, and kanamycin, and will be grown microaerophilically. Colonies will be picked and streak plated for isolated colonies. Whole cell lysates of several putative mutants grown to stationary phase will be analyzed by Western blot, probing with the anti-adhesin antiserum. Colonies not expressing Lbb1 will be selected for further analysis.

Mucosal adherence assay. The ability of *H. pylori* Lbb1 null mutants to bind to human gastric mucous cells will be compared with that of wild type *H. pylori*. This will be done using the *in situ* adherence assay described in Aim II (Falk *et. al.*, 1993). Uninfected human stomach samples expressing Le^b will be fixed in 10% formalin and embedded in paraffin. Stationary phase wild type and *lbbA*$^-$ *H. pylori* will be FITC-labeled as previously described. Tissue sections will be deparafinized and will be rinsed in PBS and then blocked with BSA. The labeled bacterial suspension will be incubated on the tissue section and the sample will be washed to remove unbound bacteria and will be viewed by fluorescent microscopy.

If the mutated gene is responsible for Le^b-dependent binding, then we should observe binding of the wild type but not mutant *H. pylori* to the tissue section. In the unlikely event that the mutant binds equally well to the tissue section as wild type, and since this binding has previously been shown to be Le^b-dependent (Boren *et. al*, 1993), it would either mean that there is more than one gene product that confers the binding ability, or that the gene actually responsible for the Le^b-specific binding was not the one cloned. These possibilities are addressed further in the analysis section.

Feasibility, Alternatives, and Analysis. We are confident that either the adhesin gene or an N-terminal fragment of the adhesin gene can be cloned into *E. coli*, as a number of researchers have had success with this. If the first cloning strategy is used, there is the remote possibility that the over-expression of an outer membrane protein from a multiple copy plasmid could be detrimental or even lethal to the growth of the *E. coli* cloning strain. This occasionally occurs if the localization of the protein in the outer membrane is aberrant and interferes with normal membrane functions. To partially safeguard against this in the first cloning approach, we will rely on the low level constitutive expression from the T7 promoter, rather than inducing it to obtain very high levels of expressed protein.

We are extremely confident that once we obtain the clone and insert a kanamycin-resistance marker into the coding sequence, crossing the mutated copy into the *H. pylori* chromosome will be straightforward. We have no reason to believe that an *lbbA* mutation in *H. pylori* grown *in vitro* would be lethal or detrimental to growth. This is especially true since it is likely that the adhesin is only expressed during the stationary phase of growth, as adherence to Le^b seems only to occur at that time. Additionally, while single crossover events resulting in both a mutated copy and a wild type copy of a given gene occasionally occurs in various systems, it seems not to be a problem in *H. pylori*. Not only have several investigators successfully obtained *H. pylori* mutants by allelic replacement, but experts in the field agree that single crossover events seem not to occur in the organism (P. Bauerfeind, H. Mobley, S. Drazek, J. Gilbert, personal communications). Of course, resulting kanamycin-resistant colonies of *H. pylori* must still be analyzed for adhesin expression before we could be certain that a mutant was obtained.

As mentioned, there is the possibility that the mutant obtained following the second cloning approach may still be able to bind gastric epithelial tissue expressing Le^b. If the second cloning approached is used, and the adhesin is a multi-subunit structure, it is possible that the subunit gene that was selected for cloning and mutagenesis may encode a gene product that interacts with the true adhesin, but is not necessary for *H. pylori* adherence. In this case we would have to select an alternate adhesin subunit for cloning and mutagenesis. In the case that more than one gene product confers binding ability, a double mutant would need to be made. This would be achieved by following the same purification, cloning, and mutagenesis schemes, but starting with the single mutant rather than wild type *H. pylori*.

If the described cloning and mutagenesis methods are not successful, we could utilize a random shuttle mutagenesis approach. Briefly, in this approach, a complete *H. pylori* genomic library would be generated and would be randomly mutagenized in *E. coli*. The entire mutated library would then be crossed into *H. pylori* and *H. pylori*

recombinants which could still bind Le^b would be absorbed out of the pool. In this way we would select for mutants that could not bind the Le^b receptor. This is not the method of choice because determining which gene has been mutagenized can be extremely time consuming. There is also the complication that the mutation may not be in a gene encoding the adhesin, itself, but in a global export or regulatory gene. For these reasons, we will try to avoid this type of mutagenesis at this time, although it would be quite useful for future studies where we would be interested in understanding regulation and export of the adhesin.

Specific Aim IV. Evaluation of the colonization of *H. pylori* in the transgenic mouse model and effects of the Lewis^b-specific adhesin mutant in this system

By this point, it will be clear whether or not the Lewis^b binding protein plays a role in adherence to gastric-surface mucosal epithelial cells *in vitro*. It would be interesting to know if this adherence is necessary for, or important in the actual colonization of the gastric mucosa *in vivo*. Clearly it would be unethical to perform such experiments in humans because of the persistent nature of *H pylori* infection. The transgenic mouse developed by Falk *et. al* (1995), however, may provide some insight into this question.

As described previously, this transgenic mouse was transfected with an α-1,3/4-fucosyltransferase fused to a pit cell lineage specific promoter, allowing for the expression of the Lewis^b epitope on the gastric mucosa of these mice. *H. pylori* strain p466 was shown to be capable of binding gastric tissue of transgenic mice *in situ*, but not that of non-transgenic littermates. It has not yet been shown, however, whether *H. pylori* can colonize the gastric epithelium of these mice *in vivo*. Before we can investigate the role of the Lewis^b-specific adhesin in colonization in this model, we must first show colonization of wild type *H. pylori*. Mice to be used for the following experiments will be obtained from Per Falk, Washington University, St. Louis, MO.

Presence of *H. pylori* in the stomach of mice after inoculation is not a sufficient measure of colonization, nor is the mere observation of gastric pathology. Marchetti *et. al.* (1995) have shown that *H. pylori* can cause gastric pathology in wild type mice (not expressing Le^b) following an extreme regiment of inoculation. This group could not, however, show persistent infection, nor did they show bacterial adherence to gastric epithelial cells. In order to show true colonization, **attached** bacteria must be measured rather than just bacteria present in the stomach or gastric pathology alone.

Data shows that bacteria passaged once *in vivo* are better able to survive in mice than lab stocks from clinical isolates that had not recently been passaged *in vivo* (Marchetti *et. al.*, 1995). This can be explained by the fact that bacteria passed in the laboratory for many generations without selective pressure to maintain genes necessary for survival in a living host may eventually lose some of these virulence factors. By passaging the bacteria *in vivo*, bacteria that have not lost crucial virulence determinants will be enriched for. For this reason, bacteria to be used in the following experiments will be passaged once *in vivo* before use as described by Marchetti *et. al.* (1995). This applies to adhesin-mutants as well as wild type *H.*

pylori. It is unlikely that adhesin-mutants would revert to wild type because the mutation was made by a large insertion. They will, nevertheless, be tested to ensure that they did not revert.

It is thought that transmission of *H. pylori* occurs by a fecal-oral route (Sutton *et. al.*, 1995). Therefore, nine transgenic and nine non-transgenic mice will be inoculated orally by dropper-feeding them approximately 200 µl of a sugar solution containing a thick slurry of stationary phase bacteria every day for one week. Mice will imbibe this solution readily if they are previously dehydrated for several hours (P. Falk, personal communication). We feel that this method of inoculation is superior to methods that utilize a feeding tube in that feeding tubes often damage gastric tissue, possibly changing the normal course of infection. Although our method does not allow for the precise quantitation of bacteria in the inoculum that feeding tube inoculation would, we feel that the benefits of starting with an intact mucosa far outweigh this disadvantage.

Three transgenic and three non-transgenic mice will be killed by cervical dislocation at each of three time points. The time points will include 48 hours, one week, and two months. The first two time points will be to measure initial colonization, and the last time point will be to confirm persistent infection. Stomachs will be harvested from the mice, weighed, cut open, and then suspended in PBS. The mouse stomachs will be washed several times in PBS to remove unbound bacteria, and then will be homogenized in a Stomacher (Tekmar, Cincinnati, OH). Homogenates will be diluted and spread-plated onto selective medium comprised of brain-heart infusion agar, 7% horse blood, 1% IsoVitalex, 6 mg/l vancomycin, 20 mg/l nalidixic acid, and 2 mg/l amphotericin and will be incubated at 37° C under microaerophilic conditions (Goodwin *et. al.*, 1985). Colonies will be counted and the number of colony forming units isolated from the stomachs of transgenic mice expressing the Lewis^b epitope will be compared to the number of colony forming units isolated from non-transgenic littermates.

If we are able to show colonization of the transgenic mice relative to the non-transgenic mice at any of the time points, the experiment will be repeated exactly as described above, but this time the adhesin mutant of *H. pylori* will be tested along with wild type. Colonization of wild type *H. pylori* will be compared to the adhesin mutant in transgenic mice. If the adhesin is necessary for colonization in the mouse model, then the expected result would be that we observe colonization of the wild type bacteria in transgenic mice, but no colonization of wild type bacteria in non-transgenic (lacking the receptor for the adhesin) mice, and no colonization of the adhesin mutant *Helicobacter* in either the transgenic or non-transgenic mice. If the adhesin is not critical, but plays a significant role in colonization, then colonization of the adhesin mutants would be decreased in both mouse types and colonization of the wild type bacteria in non-transgenic mice would be decreased compared to colonization of wild type bacteria in transgenic mice.

Feasibility, Alternatives, and Analysis. We are confident that the wild type bacteria will be able to survive in the gastric mucosa of both transgenic and non-transgenic mice for up to several weeks based on the work of Marchetti *et. al.* (1995). The first question we are asking is whether bacterial colonization can occur in mice

expressing Le^b. The likelihood of colonization occurring in the transgenic mice is suggested by the *in situ* binding data (Falk *et. al.*, 1995).

We are hoping to show long term colonization of wild type *H. pylori* in the transgenic mouse, as seen in human infection. It is likely that there may be a delay between the time the bacteria arrive in the stomach and the time that it actually penetrates the thick mucous layer and colonizes the epithelial cells. Conversely, it is possible that due to unknown factors in the mouse model, the bacteria may be able to colonize initially, but would be unable to persist for a long period of time. By observing mouse tissue at multiple time points, we will be able to control for these issues.

Only if results of this initial experiment are as expected (*i.e.*, wild type bacteria colonize transgenic but not non-transgenic littermates), will the second phase of the experiment be attempted. If experiments aimed at analyzing the importance of the Le^b-specific adhesin in colonization indicate that the adhesin is essential for *H. pylori* colonization of gastric epithelia, then this transgenic mouse model could be useful for evaluating a vaccine aimed at blocking bacterial binding.

There is, unfortunately, the unlikely possibility that binding of wild type *H. pylori* would not occur in the transgenic mouse *in vivo*, under our experimental conditions. If this were to be the case, then we would try to optimize the inoculation conditions by raising the inoculum, inoculating more often, or inoculating for a longer period of time. It also could be useful to look at colonization at different time points. If we still do not see colonization at any time point, it could be due to some inhibitory factor that is present in the gastric mucosa of mice but not of humans and is washed away or inactivated in the *in situ* binding assay. Alternatively, there is a slight possibility that bacteria may colonize non-transgenic mice, thereby nullifying positive data. This could be an artifact of not washing the stomach tissue well enough to remove unbound material. This could be overcome by more vigorous washing. It could less likely occur by some alternate interaction between bacterial and epithelial cells that does not take place when using fixed epithelial cells. If these complications cannot be overcome, we obviously will not move on to testing the adhesin mutant strain of *H. pylori* in these mice. We may either have to be satisfied with binding data obtained *in vitro*, or another animal model, such as the less convenient, more expensive gnotobiotic piglet model (Engstrand, 1995) could be tested.

The overall goal of the research proposed here is to identify and characterize the factor responsible for the binding of *Helicobacter pylori* to the human gastric mucosa. This knowledge will help pave the way to a greater understanding of the pathogenesis of this microorganism, as well as to the development of therapeutics and preventative measures against infection. Studies beyond the scope of this proposal will include epitope mapping of the adhesin using monoclonal antibodies, investigations into the regulation and export of the adhesin by random shuttle mutagenesis, and possibly further investigations into the possibility of an adhesin-based vaccine.

LITERATURE SITED

Atlas, R. 1988. Microbiology: Fundamentals and Applications. MacMillan Publishing Company. New York.

Blaser, M., G. Perez, H. Kleanhous, T. Cover, R. Peek, P. Chyou, G. Stemmermann, A. Nomura. 1995. Infection with *Helicobacter pylori* strains possesing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res.* **55**:2111-2115.

Boren, T., P. Falk, K. Roth, G. Larson, S. Normark. 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science.* **262**:1892-1895.

Boren, T., S. Normark, P. Falk. 1994. *Helicobacter pylori:* molecular basis for host recognition and bacterial adherence. *Trends Microbiol.* **2**:221-228.

Cerda, J., F. Fowler. 1994. Fighting the fire: managing and preventing peptic ulcer disease in the multi-risk patient. *Modern Med.* **62**:40-49.

Clarke, C., W, Cowan, J. Edwards, A. Howel-Evans, R. McConnell, J. Woodrow, P. Sheppard. 1955. The relationship of the ABO blood groups to duodenal and gastric ulceration. *Br. Med. J.* **2**:643-646.

Cover, T., M. Blaser. 1992. Purification and Characterization of the Vacuolating Toxin from *Helicobacter pylori. J. Biol. Chem.* **267**:10570-10575.

Cover, T., P. Cao, C. Lind, K. Tham, M. Blaser. 1993. Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates *in vitro* and *in vivo. Infect. Immun.* **61**:5008-5012.

Doig, P., T. Trust. 1994. Identification of surface-exposed outer membrane antigens of *Helicobacter pylori. Infect. Immun.* **62**:4526-4533.

Engstrand, L. 1995. Potential animal models of *Helicobacter pylori* infection in immunological and vaccine research. *FEMS Immunol. Med. Microbiol.* **10**:265-270.

Falk, P., L. Bry, J. Holgersson, J. Gordon. 1995. Expression of a human α-1,3/ 4-fucosyltransferase in the pit cell lineage of FVB/N mouse stomach results in production of Le^b-containing glycoconjugates: A potential transgenic mouse model for studying *Helicobacter pylori* infection. *Proc. Nat. Acad. Sci.* **92**:1515-1519.

Falk, P., K. Roth, T. Boren, T. Westblom, J. Gordon, S. Normark. 1993. An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc. Natl. Acad. Sci.* **90**:2035-2039.

Goodwin, C., E. Blincow, J. Warren, C. Sanderson, L. Easton. 1985. Evaluation of cultural techniques for isolating *Campylobacter pyloridis* from endoscopic

biopsies of gastric mucosa. *J. Clin. Pathol.* **38**:1127-1131.

Harlow, E., D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press.

Hazell, S., A. Lee. 1986. *Campylobacter pyloridis* urease, hydrogen ion back diffusion, and gastric ulcers. *Lancet.* **ii**:15-17.

Hjelmeland, L., A. Chrambach. 1984. Solubilization of functional membrane-bound receptors. *In* Receptor Biochemistry and Methodology. Vol. 1. 35-46.

Hoffmann, A., R. Roeder. 1991. Purification of His-tagged proteins in non-denaturing conditions suggest a convenient method for protein interaction studies. *NAR.* **19**:6337-6338.

Joiner, K., K. Warren, E. Brown, J. Swanson, M. Frank. 1983. Studies on the mechanism of bacterial resistance to complement-mediated killing. IV. C5b-9 forms high molecular weight complexes with bacterial outer membrane constituents on serum-resistant but not on serum-sensitive *Neisseria gonorrhoeae. J. Immunol.* **131**:1443-1451.

Joiner, K., K. Warren, M. Tam, M. Frank. 1985. Monoclonal antibodies directed against gonococcal protein I vary in bactericidal activity. *J. Immunol.* **134**:3411-3419.

Leying, H., S. Sauerbaum, G. Geis, R. Haas. 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. *Mol. Microbiol.* **6**:2863-2874.

Lindberg, F., B. Lund, S. Normark. 1984. Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells. *EMBO.* **3**:1167-1173.

Lindberg, F., B. Lund, L. Johansson, S. Normark. 1987. Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. *Nature.* **328**:84-87.

Lingwood, C., G. Wasfy, H. Han, M. Huesca. 1993. Receptor affinity purification of a lipid-binding adhesin from *Helicobacter pylori. Infect. Immun.* **61**:2474-2478.

Lund, B., F. Lindberg, B. Marklund, S. Normark. 1987. The PapG protein is the α-D-galactopyranosyl-(1-4)-β-D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli. PNAS.* **84**:5898-5902.

Marchetti, M., B. Arico, D. Burroni, N. Figura, R. Rappuoli, P. Ghiara. 1995. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science.* **267**:1655-1658.

Marshall, B., J. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastric and peptic ulceration. *Lancet.* **1**:1311-1315.

Mentis, A., C. Blackwell, D. Weir, C. Spiliadis, A. Dailianas, N. Skandalis. 1991. ABO blood group, secretor status and detection of *Helicobacter pylori* among patients with gastric or duodenal ulcers. *Epidemiol. Infect.* **106**:221-229.

O'Toole, P., M. Kostrzynska, T. Trust. 1994. Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. *Mol. Micro.* **14**:691-703.

Papini, E., M. Bugnoll, M. De Bernard, N. Figura, R. Rappuoli, C. Montecucco. 1993. Bafilomycin A1 inhibits *Helicobacter pylori*-induced vacuolization of HeLa cells. *Mol. Micro.* **7**:323-327.

Parsonnet, J. 1993. *Helicobacter pylori* and gastric cancer. *Gastroenterol. Clin. North Am.* **22**:89-104.

Paruchuri, D., H. Seifert, R. Ajioka, K. A. Karlsson, M. So. 1990. Identification and characterization of a *Neisseria gonorrhoeae* gene encoding a glycolipid-binding adhesin. *PNAS.* **87**:333-337.

Phizicky, E., S. Fields. 1995. Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* **59**:94-123.

Saadi, A., D. Weir, I. Poxton, J. Stewart, S. Essery, C. Blackwell, M. Raza, A. Busuttil. 1994. Isolation of an adhesin from *Staphylococcus aureus* that binds Lewis^a blood group antigens and its relevance to sudden infant death syndrome. *FEMS Immun. Med. Micro.* **8**:314-320.

Sambrook,J., E. Fritsch, T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press.

Sanger, F., S. Nicklen, A. Goulson. 1977. DNA sequencing with chain-terminating inhibitors. *PNAS.* **74**:5463-5467.

Segal, E., J. Shon, L. Tompkins. 1992. Characterization of *Helicobacter pylori* urease mutants. *Infect. Immun.* **60**:1883-1889.

Smith, A., B. Chahal, G. French. 1994. The human gastric pathogen *Helicobacter pylori* has a gene encoding an enzyme first classified as a mucinase in *Vibrio cholerae. Mol. Microbiol.* **13**:153-160.

Sutton, L., W. Wilke, N. Lynch, R. Jones. 1995. *Helicobacter pylori*-containing sewage detected by an automated polymerase chain reaction amplification procedure. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology. Washington, DC.

Talley, N. 1993. Epidemiology of *Helicobacter pylori. Gastroenterology Medicine Today.* **(suppl)**:8-13.

Yuen, S., A. Chui, K. Wilson, P. Yuan. 1989. Microanalysis of SDS-PAGE

electroblotted proteins. *Biotechniques*. **7**:74-82.

APPENDIX

Time Table

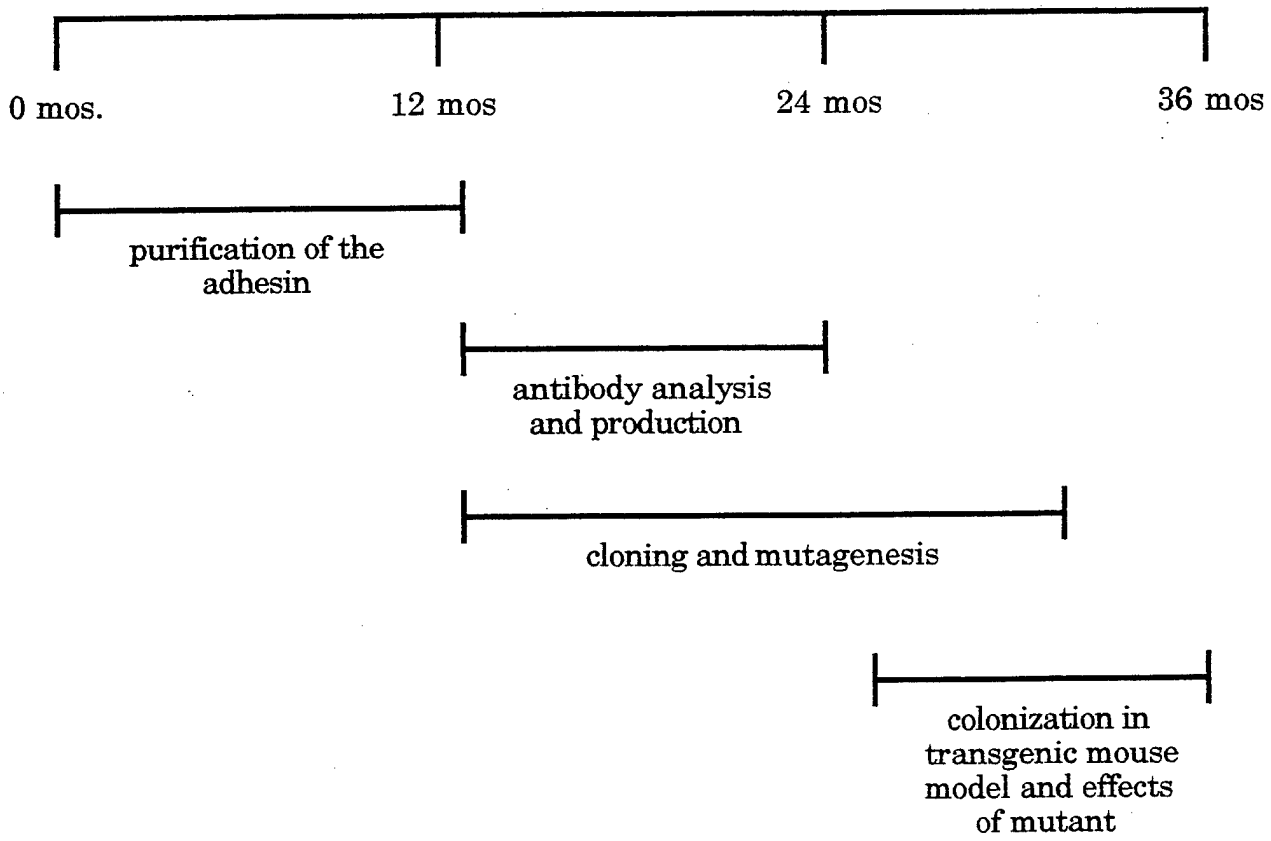


Figure 1. Lewis Blood-Group Determinants and their Precursors

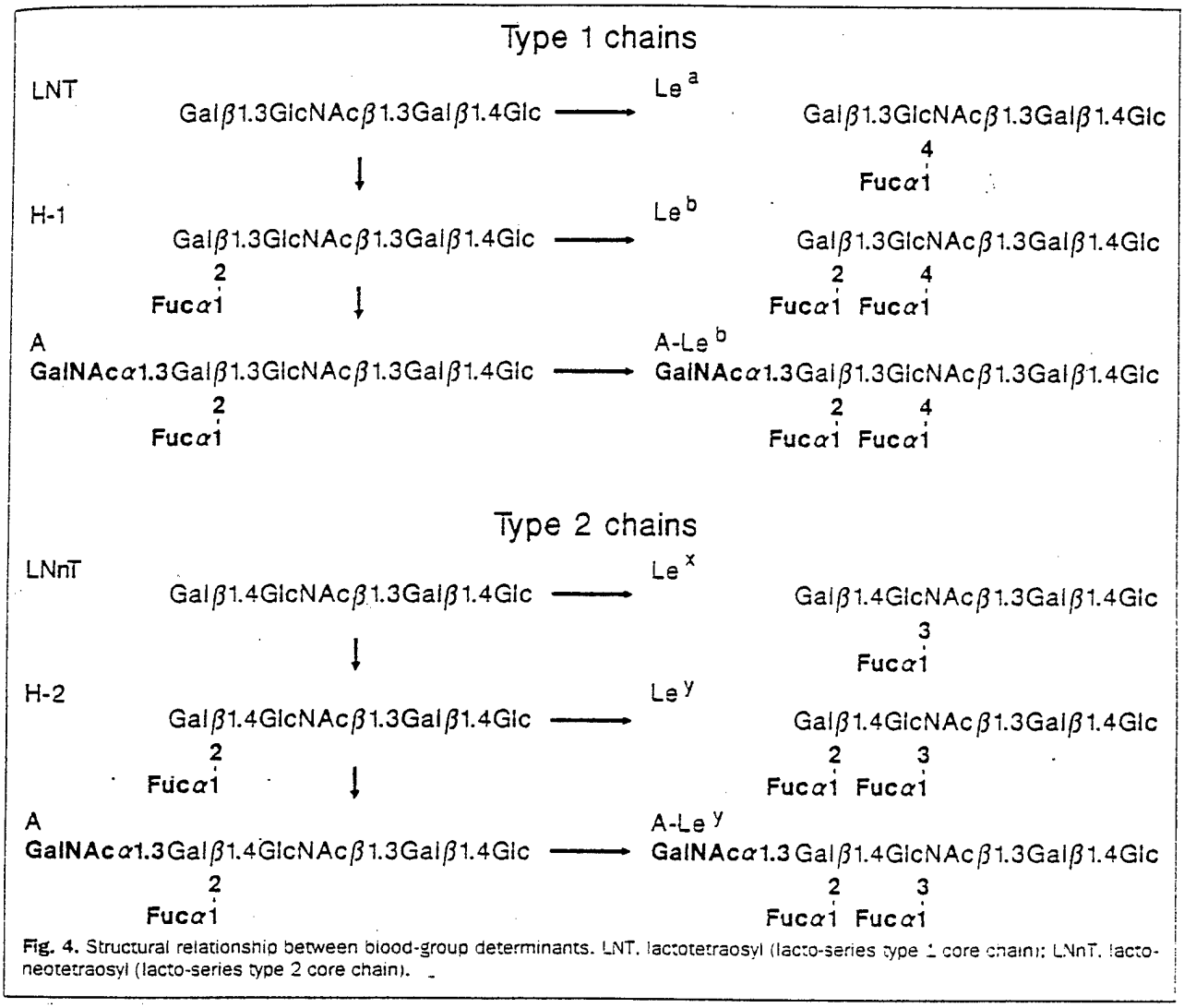
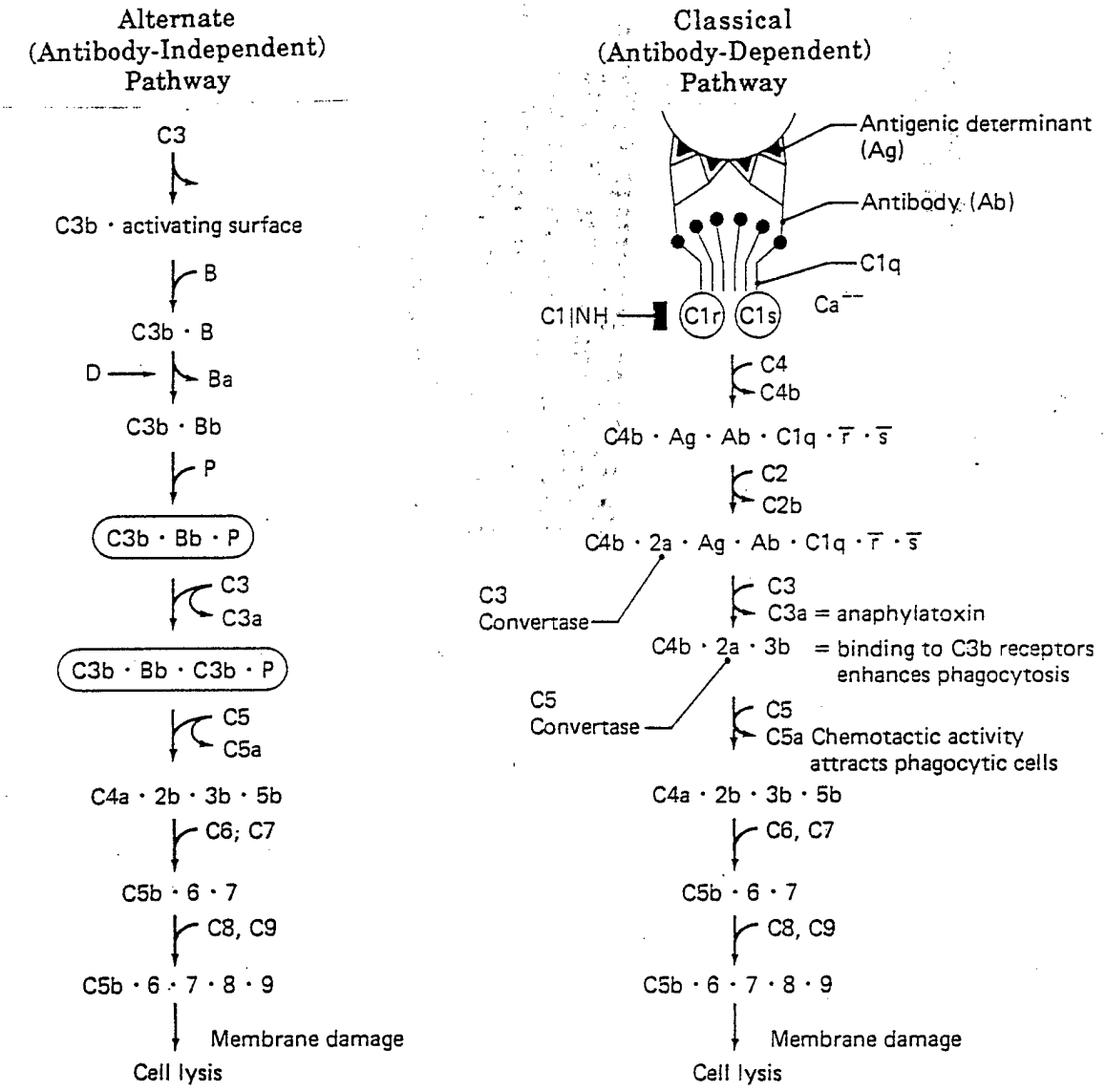


Fig. 4. Structural relationship between blood-group determinants. LNT, lactotetraosyl (lacto-series type 1 core chain); LNnT, lacto-neotetraosyl (lacto-series type 2 core chain).

from Boren *et. al.*, 1994

Figure 2. Pathways of Complement-Mediated Bacterial Killing



adapted from Atlas, 1988